IN VITRO REPRESSION OF PHAGE λ DNA TRANSCRIPTION BY A PARTIALLY PURIFIED REPRESSOR FROM LYSOGENIC CELLS*

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When phage λ infects a sensitive cell, one of two possible responses can ensue: (1) the *lytic response*, in which the cell lyses and releases new virus particles, or (2) the *lysogenic response*, in which the cell survives with the λ DNA integrated into the host DNA and replicating with it—the prophage state.¹ The lysogenic cell is characterized by a repression of lytic functions of the λ prophage and also by the ability to repress lytic development of a superinfecting λ phage.

The genetic control of prophage repression and superinfection repression has been analyzed by two types of experiments: characterization of λ mutants unable to carry out effective repression,² and the study of a recombinant between λ and the related phage 434 which probably contains all λ genes except for the small region surrounding the c_I gene (labeled *i* on Fig. 1).³ The work with λ

FIG. 1.—The vegetative genetic map of λ phage obtained from crosses during lytic growth. Genes A–R (upper-case letters) are required for the production of λ phage in a lytic response to infection.²⁰ The repression genes c_I , c_{II} and c_{III} ,² the integration genes *intA* and *intB*,²¹ and some part of the *b2* region²² are required for an effective lysogenic response to infection. The *red* gene is concerned with vegetative genetic recombination.²³ The functional clustering of the λ genes is indicated below the vegetative map. Evidence for this functional role has been summarized by Echols and Joyner.¹ The *i*-region responsible for the specificity of repression includes the c_I repressor gene and presumably additional genetic material on either side between c_I and *N* and between c_I and c_{II} .^{3, 24}

point mutants has identified three λ genes required for repression and subsequent lysogeny after infection of nonlysogenic cells: c_I , c_{II} , c_{III} (Fig. 1). Of the three, however, only the c_I gene is required for the maintenance of lysogeny and for superinfection repression. Superinfection experiments involving the λ -434 recombinant phage (λi^{434}) have shown that λi^{434} has the ability to repress 434, but not λ , and the ability to be repressed by 434, but not by λ . These experiments show that the c_I gene is required for the maintenance of prophage repression and for superinfection repression, and that the sites of action of the c_I product essential to block phage production are within the *i* region of Figure 1. Within the framework of the Jacob-Monod model⁴ for gene regulation, the c_I gene directs the synthesis of a repressor which acts on an operator site or sites within the *i* ^{λ} region; the recombinant phage λi^{434} carries the genetic determinants for the 434 repressor and its operator site(s).

Analysis of superinfection repression at a biochemical level has shown that a repressed superinfecting phage produces a much lower level of λ mRNA than a

nonrepressed phage⁵ and has indicated that all of this mRNA repression is controlled by the c_I product acting on sites within the i -region.⁶ The simplest interpretation of these results is that the c_I repressor acts to block transcription of λ DNA into mRNA—the mechanism of regulation proposed by Jacob and Monod.⁴ Ptashne has recently provided support for this view by the demonstration that the c_I -gene product binds to λ DNA, but not to λi^{434} DNA.⁷

The experiments reported in this paper demonstrate *in vitro* regulation of mRNA production in a partially purified system. Since alternative possibilities for regulation can be rendered unlikely in this system, the results provide strong support for the hypothesis that repressors act by a specific inhibition of mRNA synthesis.

Materials and Methods.—*Bacterial and phage strains and their growth:* The *E. coli* K12 strain used in this work was W3104,⁸ either nonlysogenic or lysogenic for λ^+ or λind^- . The λ phage mutant strains used were the c_I gene repression-defective mutant λc_{I1}^2 and the c_I mutant λind^- ,⁹ which as a prophage is not induced to lytic development by UV irradiation. Growth of cells and phage stocks and phage infection were carried out as previously described.⁵

DNA preparation: Pure λ DNA for hybridization and some repression experiments was prepared by phenol treatment of λ phage which had been purified by CsCl density-gradient centrifugation.¹⁰ The mixture of λ and *E. coli* DNA used for most repression experiments was prepared by CsCl density-gradient centrifugation of crude lysozyme lysates¹¹ of W3104 (λ^+) which had been infected by λc_{I1} .

Repressor preparation: W3104 (λind^-) was used as repressor source since the “non-inducible” character of λind^- suggested a more plentiful repressor supply or a more stable repressor. Experimental support for the latter idea has been presented by Ptashne.⁷ A 3000-ml culture of W3104 (λind^-) or nonlysogenic W3104 (5×10^8 cells/ml) was chilled, centrifuged, and washed by centrifugation in 0.05 *M* tris-HCl, pH 7.9. The pellet was frozen and thawed and then resuspended in 30 ml 0.05 *M* tris-HCl, pH 7.9, containing 12% sucrose. The cell suspension was then warmed for 1 min in a 37°C bath and treated with 200 μ g/ml lysozyme in the presence of 0.002 *M* EDTA for 5 min at 37°C; $MgCl_2$, $MnCl_2$, and β -mercaptoethanol were added to concentrations of 0.06 *M*, 0.015 *M*, and 0.006 *M*, respectively, and lysis was completed by a 2-min incubation with the nonionic detergent Triton X-100 (Ruger, Irvington, N.Y.) at a concentration of 1.3%.¹² Streptomycin was added to the chilled lysate to a concentration of 0.75%, and the precipitate removed by centrifugation at $10,000 \times g$ for 20 min. Subsequent operations were carried out at 0–4°C. The majority of the streptomycin and other small molecules were removed from the supernatant fraction by passage over a Sephadex G-50 coarse column (Pharmacia, Uppsala), and this “Sm-Sephadex fraction” was stored in liquid nitrogen (in 0.01 *M* tris-HCl, pH 7.4, 0.005 *M* $MgCl_2$, and 0.01 *M* β -mercaptoethanol—designated as TMM buffer). Further fractionation was carried out by centrifugation at $100,000 \times g$ for 120 min; the pellet (“ribosome fraction”) was resuspended in 0.01 *M* tris-HCl, pH 7.9, and 0.01 *M* $MgCl_2$, and the upper two thirds of the supernatant fraction was purified further by $(NH_4)_2SO_4$ precipitation. After centrifugation of a 40% saturated $(NH_4)_2SO_4$ solution, the supernatant fraction was made 60% saturated, and the precipitate (“40–60 $(NH_4)_2SO_4$ fraction”) was collected by centrifugation, redissolved in TMM buffer, and dialyzed against the same buffer. Here “40% saturated” means that 40 ml of an $(NH_4)_2SO_4$ solution (saturated at 26°C) is added per 100 ml of total solution. The 40–60 $(NH_4)_2SO_4$ fraction was applied to a DEAE-cellulose column, and successive “batch” elutions carried out by washing with TMM + 0.01 *M* KCl, TMM + 0.15 *M* KCl, TMM + 0.30 *M* KCl, and TMM + 1.0 *M* KCl. Protein-containing fractions from each salt concentration were pooled, precipitated in 70% saturated $(NH_4)_2SO_4$, and the precipitate redissolved in TMM and dialyzed against TMM buffer to remove residual $(NH_4)_2SO_4$.

RNA synthesis, purification, and hybridization: RNA synthesis was carried out using conditions similar to these of Chamberlin and Berg.¹³ One-ml reaction mixtures contained 0.01 *M* tris-HCl, pH 7.9, 0.008 *M* MgCl₂, 0.002 *M* MnCl₂, 0.005 *M* β -mercaptoethanol, normally 10 μ g DNA, 1 mg ribosomes, repressor fractions, 400 μ moles ATP, GTP, UTP, 80 μ moles H³-CTP (Schwarz, 1.4 mc/ μ mole), and 6 units RNA polymerase. RNA polymerase was purified as described by Chamberlin and Berg¹³ except that *E. coli* W3104 cells were used instead of *E. coli* B. The specific activity of the RNA polymerase preparation was approximately 1000 units/mg. RNA synthesis was carried out for 5 min at 37°C; the rate of RNA synthesis in this system was constant for at least 10 min (as measured by incorporation of H³-CTP into TCA-insoluble material). The repressor fraction (except where noted) was added at 0°C 2 min before the RNA polymerase and triphosphates. RNA was purified by hot phenol essentially as described previously:⁵ the 1-ml reaction mixture was warmed to 37°C for 1 min in the presence of 0.001% macaloid and 2.5% sodium dodecyl sulfate, diluted to 2 ml with "C buffer" (0.01 *M* tris-HCl, 0.01 *M* potassium citrate, 0.02 *M* KCl, pH 7.3), and shaken for 3 min at 60°C with an equal volume of phenol; the phenol treatment of the aqueous phase was repeated, and the resultant RNA preparation was heated to 100°C for 5 min, cooled quickly, made 0.5 *M* in NaCl, and filtered through a Millipore filter (0.45 μ) to remove DNA; the RNA was then passed over a Sephadex G-50 fine column and used for hybridization. DNA-RNA hybridization was carried out as described previously¹⁴ in a standard 0.6-ml hybridization mixture containing 20 μ g of denatured λ DNA, 0.4 ml of the RNA preparation, and 0.06 ml phenol.

Results.—Assay and partial purification of the repressor: The object of our experiments was to study the biochemical mechanism of repression in an *in vitro* system in which the components could be separated. We assumed that repression was likely to occur by an inhibition of λ DNA transcription into mRNA, as suggested by the *in vivo* work; we therefore began with a system containing λ DNA and RNA polymerase and sought for a specific repressor of λ transcription in extracts of lysogenic cells.

We wanted an *in vitro* mRNA assay for repression which would detect only *specific* inhibition of λ transcription and be insensitive to any general inhibitors or activators of *in vitro* RNA synthesis. To accomplish this and also to begin with a form of λ DNA which we knew to be sensitive to repression, we employed as a DNA source a mixture of λ and *E. coli* DNA extracted from infected lysogenic cells. Since approximately 95 per cent of the DNA is *E. coli* DNA, most of the RNA made is transcribed from *E. coli* DNA and not from λ ; therefore we can look for a specific inhibition of λ transcription with respect to *E. coli* by a decrease in the fraction of the total RNA which will hybridize with λ DNA. As an additional check on specificity, we have carried out parallel fractionations of lysogenic and nonlysogenic extracts, so that there is also available a direct determination of the differential inhibition by the repressor-containing fraction.

Table 1 shows the use of this repression assay to demonstrate *in vitro* repression of λ mRNA synthesis by a crude fraction and to follow the repression activity through an (NH₄)₂SO₄ fractionation. We consider the best measure of specific repression to be the "degree of repression" of column 6—the percent λ RNA of the total RNA for the nonlysogenic extract divided by the per cent λ RNA of the total RNA for the lysogenic extract. The degree of repression found in this experiment was larger than usual—more typical values are approximately 2.5 for both crude and 40–60 (NH₄)₂SO₄ fractions. A surprising (to us) finding was

that effective repression at each stage of repressor fractionation required the presence of ribosomes. The nature of this requirement is explored below.

The repression activity was fractionated further by DEAE-cellulose chromatography (Table 2). Total RNA synthesis varies markedly from one fraction to another (particularly in the case of the 0.30 *M* fraction which contains the RNA polymerase activity of the extracts¹³); however, only the 0.15 *M* fraction shows a specific differential inhibition of λ mRNA synthesis. The repression activity of the 0.15 *M* DEAE-cellulose fraction has been demonstrated in six independent extracts—the degree of repression has ranged from 1.8 to 2.9. The

TABLE 1. $(\text{NH}_4)_2\text{SO}_4$ fractionation of repressor activity.

Fraction	Source	Total RNA (cpm)	λ RNA (cpm)	λ /Total (%)	Degree of repression
Sm-Sephadex	(o)	9,950	302	3.0	4.3
Sm-Sephadex	(λ)	9,940	70	0.7	
0-40(NH_4) ₂ SO ₄	(o)	6,450	158	2.4	1.0
0-40(NH_4) ₂ SO ₄	(λ)	7,300	176	2.3	
40-60(NH_4) ₂ SO ₄	(o)	12,000	295	2.5	2.8
40-60(NH_4) ₂ SO ₄	(λ)	9,800	88	0.9	
60-100(NH_4) ₂ SO ₄	(o)	5,360	87	1.6	0.9
60-100(NH_4) ₂ SO ₄	(λ)	5,080	92	1.8	

The Sm-Sephadex fraction refers to the fraction obtained after removal of DNA by streptomycin precipitation, subsequent passage over Sephadex G-50, and high-speed centrifugation to remove ribosomes (see *Methods*). The 0-40 (NH_4)₂SO₄ fraction refers to the material precipitated in an (NH_4)₂SO₄ solution at 40% of saturation; 40-60 refers to the material remaining in the 0-40 supernatant fraction which is precipitated at 60% of saturation; and 60-100 refers to the material remaining in the 40-60 supernatant fraction which is precipitated in a saturated (NH_4)₂SO₄ solution. Total RNA (cpm) refers to 0.4 ml of the purified RNA synthesized *in vitro*; λ RNA (cpm) refers to λ -specific RNA measured by DNA-RNA hybridization using 0.4 ml of the purified RNA—without DNA blank of approximately 30 cpm has been determined for each RNA and subtracted; λ /total gives the fraction of the RNA synthesized *in vitro* which is λ -specific (col. 4/col. 3); "degree of repression" is the ratio of λ /total for nonlysogenic cells to λ /total for lysogenic cells—a measurement of the extent to which λ RNA synthesis is specifically inhibited by the lysogenic cell extract. For RNA synthesis, each reaction mixture contained 10 μg DNA (λ + *coli*), 6 units of RNA polymerase, 1 mg of ribosomes (from the nonlysogenic extract), and 4 mg "repressor fractions" (6 mg for "Sm-Sephadex"). For details of RNA synthesis, RNA purification, and DNA-RNA hybridization, see *Methods*.

TABLE 2. DEAE fractionation of repressor activity.

Fraction	Source	Total RNA (cpm)	λ RNA (cpm)	λ /Total (%)	Degree of repression
40-60 (NH_4) ₂ SO ₄	(o)	13,800	320	2.3	1.8
40-60 (NH_4) ₂ SO ₄	(λ)	14,000	180	1.3	
0.01 <i>M</i> DEAE	(o)	6,560	119	1.8	1.1
0.01 <i>M</i> DEAE	(λ)	6,570	111	1.7	
0.15 <i>M</i> DEAE	(o)	11,800	342	2.9	1.9
0.15 <i>M</i> DEAE	(λ)	12,700	197	1.5	
0.30 <i>M</i> DEAE	(o)	34,700	697	2.0	1.0
0.30 <i>M</i> DEAE	(λ)	30,700	602	2.0	
1.0 <i>M</i> DEAE	(o)	8,470	202	2.4	0.8
1.0 <i>M</i> DEAE	(λ)	8,400	263	3.1	

The 40-60 (NH_4)₂SO₄ fraction was prepared by (NH_4)₂SO₄ fractionation as in Table 1. The DEAE-fractions refer to the fractions obtained by elution of the 40-60 (NH_4)₂SO₄ fraction from DEAE-cellulose at KCl concentrations of 0.01 *M*, 0.15 *M*, 0.30 *M*, and 1.0 *M*, respectively. The derivation and presentation of data are the same as for Table 1.

presence of the c_I -repressor gene product in this fraction would be expected from the previous chromatography of radioactive c_I protein by Ptashne.⁷

Characteristics of the repression system: We would like to attribute the *in vitro* repression activity described above to the product of the c_I gene. Since we know that the sites of action of the c_I repressor necessary to block *in vivo* phage production³ and transcription⁶ are within the *i*-region, we can gain presumptive evidence on this point from the use of λi^{434} DNA as the viral DNA in the *in vitro* system. DNA from λi^{434} should be insensitive to the c_I repressor (see introduction); in accord with this expectation no repression was found in the *in vitro* system when λi^{434} DNA replaced λ DNA (Table 3, lines 1 and 2).

TABLE 3. *Characteristics of the repression system.*

Repressor	DNA	Degree of repression
2 mg	$\lambda + coli$	2.9
2 mg	$\lambda i^{434} + coli$	0.8
1 mg	$\lambda + coli$	2.3
0.2 mg	$\lambda + coli$	1.0
2 mg	Pure λ (2 μ g)	2.1
2 mg (+5 min)	$\lambda + coli$	0.8
2 mg + CAP	$\lambda + coli$	2.3

The "repressor fraction" is the 0.15 DEAE-fraction of Table 2.

The " $\lambda + coli$ " and " $\lambda i^{434} + coli$ " DNA refer to DNA (10 μ g) extracted from infected cells (lysogenic for λ or λi^{434} , respectively).

"Pure" λ DNA refers to DNA extracted from phage. "Degree of repression" is determined for " $\lambda + coli$ " DNA as in Tables 1 and 2—% λ RNA of total for the nonlysogenic extract divided by % λ RNA of total for the lysogenic extract.

For pure λ DNA, degree of repression = λ RNA cpm(o)/ λ RNA cpm (λ).

For the chloramphenicol (CAP) experiment of line 5, 150 μ g/ml of CAP was present. For the "+5 min" experiment of line 6, the repressor fraction was not added until RNA synthesis had been proceeding for 5 min; incubation was then continued for an additional 5 min.

Conditions of RNA synthesis, RNA purification, and DNA-RNA hybridization were the same as for Table 1 and Table 2 (see *Methods*).

We have also studied the effect of repressor concentration and DNA source on the repression system. Repression becomes ineffective at a protein concentration approximately tenfold less than that of the standard 2 mg/ml (Table 3, lines 1–4). Purified λ DNA extracted from mature phage also appears to be sensitive to *in vitro* repression (Table 3, line 5).

All of the data presented so far have supported the concept that repression occurs by a specific inhibition of RNA synthesis. Two major alternatives to this concept have been proposed: (1) that repression occurs by an inhibition of protein synthesis and that mRNA production is affected because continued transcription requires mRNA translation;¹⁵ (2) that repression occurs by a specific destruction of mRNA rather than specific inhibition of synthesis.¹⁶ Although we cannot absolutely rule out these hypotheses in our system, we can render them unlikely.

If the destruction (nuclease) model were correct, then delayed addition of the nuclease repressor and further incubation might be expected to produce some degree of repression. Table 3 (line 6) shows, however, that addition of repressor after five minutes of RNA synthesis and further incubation at 37°C for five

minutes leads to no repression. Complex destruction models can of course be imagined which are consistent with our data.

If the "coupled transcription-translation" model were correct, then protein synthesis should be required in order for controlled RNA synthesis to occur. In spite of the fact that ribosomes seem to be necessary in our system for effective repression, we think that this coupled model is extremely unlikely for three reasons: (1) the protein synthesis inhibitor chloramphenicol has no effect on the repression (Table 3, line 7); (2) the ribosome requirement is not an absolute one—some repression is observed in the absence of ribosomes (Table 4A); (3) very little protein synthesis occurs relative to nucleic acid synthesis in our repression system, as expected on the basis of its prior purification (Table 4B).

The extent of protein synthesis in the repression system was estimated by the addition of a small quantity of purified H^3 -phe-tRNA (~ 0.3 tRNA/ribosome) to the repression system under conditions of RNA synthesis. Since amino acids should have been removed from the system by Sephadex treatment, any protein synthesis occurring would likely arise from aminoacyl-tRNA bound to the ribosomes (free tRNA does not elute from DEAE until the 1.0 M fraction). We therefore added the purified H^3 -phe-tRNA to the system to measure the extent

TABLE 4. *Role of the ribosome and protein synthesis in repression.*

(A) *The ribosome:*

Repressor fraction	Ribosomes	Total RNA synthesis (cpm/0.25 ml)	λ RNA (% of total)	Degree of repression
(o)	+	65,700	6.1	2.0
(λ)	+	57,000	3.0	
(o)	—	73,000	4.0	1.3
(λ)	—	63,300	3.1	

(B) *Protein synthesis:*

Addition	Cold TCA (cpm/0.25 ml)	Hot TCA (cpm/0.25 ml)
H^3 -phe-tRNA	255	15
H^3 -phe-tRNA + tRNA system	230	114
H^3 -phe-tRNA (no incubation)	562	4

The "repressor fraction" is the 0.15 DEAE-fraction of Table 2. Total RNA synthesis is measured by a precipitation with cold 5% TCA at the end of the 5-min period of RNA synthesis. The % λ RNA was measured as for Table 1 and Table 2. The degree of repression is the same nonlysogenic-to-lysogenic ratio of Tables 1–3. In Table 4B, H^3 -phe-tRNA refers to purified tRNA for phenylalanine; 3 μ g was added per 1 ml incubation mixture (~ 0.3 tRNA/ribosome). The "tRNA system" refers to a mixture of tRNA carrying 20 unlabeled amino acids (100 μ g/ml), 19 of the 20 standard amino acids (no phe) at 2×10^{-4} M , and a crude aminoacyl synthetase fraction (SFII, 400 μ g/ml).¹⁷ Cold TCA (cpm) is the amount of radioactivity precipitated by cold 5% TCA at the end of the standard 5-min incubation at 37° used for RNA synthesis—a measure of protein synthesis plus remaining H^3 -phe-tRNA; hot TCA (cpm) is the amount of radioactivity precipitated by 5% TCA after the incubation mixture was boiled for 5 min in 5% TCA—a measure of protein synthesis. For the protein synthesis experiment (Table 4B, lines 1 and 2), incubation conditions were the same as for the RNA synthesis experiments except that 80 $m\mu$ moles/ml of nonradioactive CTP was used instead of the usual H^3 -CTP. Table 4B, line 3, gives the results of TCA precipitations without the 37°C incubation. Equal amounts of H^3 -phe-tRNA were added for all three experiments. In an unfractionated protein-synthesizing system (Sm-Sephadex) without added DNA, but with added R17 viral RNA and the above amino acid mixture, 75% of the H^3 -phe was incorporated from H^3 -phe-tRNA into hot TCA-insoluble material. The results given in Table 4B are for a 0.15 M DEAE-fraction from nonlysogenic cells; the results for a lysogenic extract are not significantly different. The specific activities of the H^3 -CTP and H^3 -phe were 1.4 mc/ μ mole and 2.5 mc/ μ mole, respectively.

of such protein synthesis without adding a complete complement of aminoacyl-tRNA which might support further synthesis. No more than 20 cpm were incorporated into hot TCA-insoluble material (compare lines 1 and 3 of Table 4B), although the H^3 -phe-tRNA was active in a more complete protein-synthesizing system (Table 4B, line 2). From a comparison of the amino acid and nucleotide incorporation (Table 4A and B), we can estimate that protein synthesis (at least into acid-insoluble peptides) occurs at a level of ≤ 1 phe/-7000 CTP. In another experiment in which free H^3 -phe was added at the same high specific activity, less than 10 cpm were incorporated into acid-insoluble peptides out of 100,000 cpm added.

Since there is no evidence for a transcription-translation coupling, we think that the likely role of the ribosomes in *in vitro* repression is simply to bind and remove the RNA product of the RNA polymerase reaction and therefore facilitate better synthesis under derepressed conditions. In Table 4A, the poorer degree of repression without ribosomes results from less λ mRNA synthesis by the nonlysogenic system; this has been a consistent characteristic of the ribosome-deficient reaction. Decreased total RNA synthesis has not generally been found in the ribosome-deficient reaction, however, and it seems likely that under our conditions other factors normally limit the rate of mRNA synthesis from *E. coli* DNA. A stimulation by ribosomes of RNA synthesis from a DNA template has been previously observed by Shin and Moldave.¹⁸

Discussion and Summary.—Our experiments have demonstrated *in vitro* repression of λ mRNA production in a partially purified system—a finding consistent with the concept that repressors act to block transcription of DNA into mRNA.⁴ We have also provided evidence suggesting that the most popular alternative models for regulation are not correct (at least for λ). Ptashne has previously demonstrated that the c_I repressor binds specifically to the λi -region. Considering RNA synthesis and binding results together, there appears to be very strong evidence that the c_I repressor acts by an inhibition of λ mRNA synthesis. Repressor binding experiments by Gilbert and Müller-Hill in the *E. coli lac* system¹⁹ suggest strongly that repressors act at the DNA level in bacteria as well.

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Abbreviations: EDTA, ethylenediaminetetraacetate; ATP, adenosine-5'-triphosphate; CTP, cytidine-5'-triphosphate; GTP, guanosine-5'-triphosphate; UTP, uridine-5'-triphosphate; DEAE, O-(diethylaminoethyl).

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